CALBIRATION OF PHENOL OXIDASE MEASUREMENT IN ACIDIC WETLAND ENVIRONMENTS

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CALIBRATION OF PHENOL OXIDASE MEASUREMENT IN ACIDIC WETLAND ENVIRONMENTS

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LIST OF SYMBOLS AND ABBREVIATIONS

ABTS	2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
L-DOPA	L-3,4-dihydroxyphenylalanine
PO	Phenol Oxidase

SUMMARY

Phenol oxidases mediate the degradation of recalcitrant compounds, polyphenolics, in wetland soils and are considered to play a key role in the microbial carbon cycle of peatlands which predominate in boreal biomes. In order to validate a method for quantification of oxidative enzyme activity in acidic wetland environments, the relationship between pH and substrate oxidation was studied using the standard enzyme tyrosinase and in soils collected from six freshwater wetlands including three marshes in north Florida and peatlands of northern Minnesota. Phenol oxidase (PO) activity was quantified with two commonly used assay substrates, ABTS (2,2'-azino-bis(3-ethylobenzthiazoline-6-sulfonic acid) and L-DOPA (L-3,4-dihydroxyphenylalanine), across a pH range of 4 to 7 which matched the in situ pH range of the studied wetlands. The PO assay is sensitive and activity could be detected with either substrate across a pH range of 4 to 7. However, with the standard enzyme tyrosinase, it was shown that a large change or threshold in oxidation rates occurred at pH 5. At pH < 5, L-DOPA oxidation rates were greatly diminished and ABTS oxidation was at a maximum. Above pH 5, ABTS oxidation occurred at much slower rates and L-DOPA oxidation was at a maximum. The pH response of PO activity in wetland soils corroborated observations made with tyrosinase. Thus, ABTS is recommended to be an effective substrate for the quantification of PO activity at an in situ pH of < 5, while L-DOPA is recommended at an in situ pH of > 5. In soils collected from a northern Minnesota peatland, assays conducted at an in situ pH of 4 showed one to two orders of magnitude higher rates of PO activity in solid phase peat in comparison to porewater, indicating that the majority of PO activity is associated with the peat. At three Minnesota peatland sites, PO activity was

shown to attenuate with depth in agreement with the activities of other enzymes and with rates of peat decomposition.

INTRODUCTION

Boreal or northern peatlands sequester one-third of all soil carbon and currently act as major sinks of atmospheric carbon dioxide (Gorham, 1991). The ability to predict or to simulate the fate of stored carbon in response to climatic disruption remains hampered by our limited understanding of the controls of carbon turnover and the composition and functioning of peatland microbial communities. Given their global extent and uncertain fate with climatic change, peatlands are considered a high priority for climate change research.

Carbon storage in the peat is largely attributed to extremely low decomposition rates, and mechanisms proposed to account for the slow decomposition include the effects on microbial metabolism of environmental conditions (low temperature, oxygen availability, pH, nutrient supply; Limpens et al., 2008) along with the inhibitory effects of plant-derived polyphenolic compounds (Freeman et al. 2001). Extracellular enzyme hydrolysis is considered to be the rate-limiting step in the overall decomposition process in wetlands (McLatchey and Reddy 1998; Sinsabaugh et al. 1993). Seminal work by Freeman and colleagues (2001) observed that the anaerobic conditions in peatlands prevent the enzyme phenol oxidase (PO) from eliminating phenolic compounds that inhibit biodegradation. In addition, degradation or removal of phenolic compounds by PO was shown to have downstream effects on hydrolytic enzyme activity (Freeman et al. 2004), leading to the elevated release of greenhouse gases (Fenner et al., 2011). Thus, this single class of peatland enzymes, phenol oxidases, is likely to play a key role in the release of a major store of global carbon into the atmosphere, with potentially serious implications for future global climate. Insight into environmental controls and function of

phenol oxidase (PO) enzymes is critically important to our understanding of the global carbon cycle.

Phenol oxidases are a class of copper-containing oxidoreductase enzymes that require molecular oxygen for catalytic activity (Claus, 2004). Phenol oxidase, is a general enzyme class that includes laccase and tyrosinase. These enzymes utilize oxygen or peroxides as an electron acceptor to initiate the decomposition of recalcitrant polyphenolic compounds derived from lignin or lignin-like compounds (Sinsabaugh, 2010). Phenol oxidase enzymes are produced by all microbial domains of life and are found in a multitude of natural and managed ecosystems (Claus 2004). The broad target specificity of these oxidative enzymes creates difficulty in elucidation of the mechanisms and controls of phenolic compound degradation (Baldrian, 2006).

Oxidative enzymes such as PO are understudied in comparison to other extracellular enzymes such cellulase, phosphatase, and hydrolase (Sinsabaugh, 2010). The majority of previous work on oxidative enzymes has focused on upland forest soils and other soil environments, which are not saturated with water (Sinsabaugh et al., 2010; Bach et al 2013). Less information is available from wetland ecosystems, and acidic wetlands are the rule rather than the exception in boreal zones (Holden, 2005; Gorham, 1991; Post et al., 1982, 1985).

Although extracellular enzymes have been studied in soils for over a half-century, our understanding of the role of microbial enzymes in the carbon cycle remains hindered by methodological challenges. The activity of oxidative enzymes is difficult to quantify due to the complex interactions that occur between the enzymes, assay substrates, and the soil matrix, including non-specific free radical reactions mediated by PO in the presence

of oxygen (Bach et al., 2013). L-DOPA (L-3,4-dihydroxyphenylalanine), with a pH optimum near neutral (Pind et al., 1994), is by far the most commonly used assay substrate for oxidative enzymes (Sinsabaugh, 2010). However, past studies reveal that the rate of L-DOPA oxidation may not be linear with time, substrate concentration, or soil dilution (Sinsabaugh, 2010), and thus differences between experimental assay conditions and field conditions can result in unrealistic PO activities. In particular, changes in soil pH were shown to result in a variable or unrealistic PO determination (Bach et al., 2013; Tahvanainen and Haraguchi, 2013).

Although past studies have called for standardization and calibration of oxidative enzyme methods between researchers and laboratories (German et al., 2011), interpretation of assay results remains difficult, especially in understudied acidic, wetlands. These enzymes have been implicated in the global carbon cycle and climate change, but the biogeochemical controls of their activity require further research in critical, high-carbon environments. Therefore, the objective of this study was to verify and calibrate PO assay methods for use in acidic wetlands with particular emphasis on boreal peatlands due to their relative vast carbon stores. Phenol oxidase activity was quantified using the purified enzyme, tyrosinase, as well as in the soils of five wetlands across an in situ pH range of 4 to 7. Assay substrates L-DOPA and ABTS (2,2'-azino-bis(3-ethylobenzthiazoline-6-sulfonic acid) were compared across a range in pH. Based on the optimal pH for the oxidation of assay substrates, it was determined that the use of ABTS will more accurately represent oxidative enzyme activity at the in situ pH of 4 observed in boreal wetlands.

The overall goal of this research is to understand carbon turnover and greenhouse gas release in northern peatlands. Thus, the PO assay was applied to bog and fen environments in peatlands of northern Minnesota. It was hypothesized that 1.) pH will have a significant effect on potential PO activity regardless of site, 2.) potential PO activity would attenuate with depth as peat was decomposed, and 3.) potential activity would parallel with in situ temperature changes across seasons.

METHODS:

Sample collection:

Soil samples for preliminary experiments were collected during multiple field excursions from six freshwater wetland systems (Table 1). Sample sites included two sites, S1 bog and Bog Lake fen, within Marcell Experimental Forest (MEF) in northern Minnesota. The S1 bog is an acidic (pH≈3.5-4.0) and nutrient-deficient environment that receives water inputs primarily from precipitation, and is mainly covered by Sphagnum moss, shrubs, black spruce, and eastern tamarack. In contrast, the Bog Lake fen (pH 4.5-4.8) is a poor fen, which is influenced by inputs of more nutrient-rich groundwater and is dominated by Sphagnum moss and sedges. Site hydrological and vegetation information is described in more detail elsewhere (Sebestyen et al., 2011; http://mnspruce.ornl.gov). At MEF, peat cores were sampled in low elevation areas (hollows) where the water level fluctuates seasonally from the surface to approximately 20-30 cm below the top of the Sphagnum layer. Pore water samples were collected using piezometers and a peristaltic pump in parallel with peat cores during the July 2012 trip as described previously (Tfaily et al., 2014; Lin et al., 2014). Cores from each peatland site were sectioned into increments of 0-20, 30-50, and 50-100 cm intervals. Each section was homogenized in a sterile bag and subsampled for microbiological and geochemical analyses. For seasonal and MN field site comparisons, Zim bog was added as a sample site due to its proximity and similarity in nutrient inputs to S1 bog. Core samples for seasonal comparison of S1, Bog Lake fen and Zim Bog were collected in similar fashion in July 2013 and in April 2014. Seasonal comparisons were made to assess any differences in activity that could be attributed to cold temperatures, frozen conditions and nutrient differences in a non-

growing season. Triplicate cores were taken from each field site for pH response experiments and five cores per site were sampled in the field for seasonal comparisons of potential activity.

Coring is a destructive sampling method and non-destructive sampling methods are needed for in situ climate change experiments in order to avoid irreparable damage to field sites. Thus, destructive (coring) and non-destructive (claw, porewater) methods were compared in the Minnesota peatlands. All samples were stored at 4 ^{o}C in the dark and analyzed within one week of collection.

Soils from Florida wetlands were sampled using similar methods as those described above, with the exception that only the 0 to 20 cm depth interval was interrogated for PO activity. Sites (319 marsh, Center Swamp and Panacea Marsh labeled as 319, CS, PM, respectively) were located near highway 319 in northern Florida. Both the 319 Marsh and Center Swamp contained sandy textured soil covered with several centimeters of decaying leaf material and were primarily covered by cypress (*Taxodium distichum)*. Panacea marsh sediment had a smooth muddy texture and was primarily comprised of decaying cattails *(Typha lattifolia)*. Three cores were taken for pH comparisons from each Florida field site. Florida sites were chosen due to ease of sampling access, proximity and due to spread of pH at environmental conditions.

Site ID	SUVA (aromaticity)	DOC (mM)	pH	salt content (ppt)	Location
319 Swamp	2.43	5.67	4.7	2.0	30.19^{o} N, 84.19o W
Center Swamp	2.27	3.89	4.8	2.0	30.18o N, 84.21' W
Panacea Marsh	3.29	6.79	7	3.0	30.0o N, 84.23o W
S1 Bog	3.97	6.50	4	0.0	47.56o N, 93.4o W
Bog Lake fen	3.90	3.33	4.5	0.2	47.56o N, 93.48o W
Zim	3.71	5.41	~4	-	47.18o N, 92.71o W

Table 1: Porewater chemistry and location of freshwater wetland study sites

Phenol oxidase assay protocol, tyrosinsase pH range experiment:

The effects of assay pH were assessed on the activity of PO enzymes utilizing L-DOPA and ABTS as substrates. The concentration of substrates was adjusted to oversaturation in order to assure that substrate limitation did not affect the assay. A standard assay protocol was employed in multiwell plates as described previously (Lin et al., 2014). Each experimental sample had six technical replicates. Response of purified enzyme tyrosinase at range of pH was measured by a colorimetric assay. Tyrosinase (Sigma) is an enzyme within the class of phenol oxidases that was purified from the fungal strain *Trichoderma reesei*. Universal buffer, comprised of 20 mM citric acid and 10 mM KH_2PO_4, was employed due to its buffering capacity at a wide range of pH (McIlvaine 1921). The pH of the homogenate was then adjusted to 4, 5, 6, or 7 with HCl or NaOH. Substrates were prepared in buffer at 10 mM and added in a 1:1 dilution to a buffered tyrosinase solution to a final concentration of 5 mM substrate in the reaction wells. Absorbance was measured in a plate reader at wavelength of 420 nm for ABTS,

460 nm for L-DOPA oxidation. Controls were utilized to assess differences in absorbance due to change in oxidative states of reagent. Control wells contained additional buffer in the place of substrate solution and reagent blanks substituted buffer in place of tyrosinsase buffer solution.

Phenol oxidase assay protocol, soil enzyme pH range experiment:

In subsequent experiments, soil homogenate was used in place of tyrosinase. One gram of soil was homogenized in 50 ml of universal buffer comprised of 20 mM citric acid and 10 mM KH_2PO_4 using a stomacher at 230 rpm for 1 minute. Each experimental sample had six technical replicates for each individual core. Wells used for experimental replicates contained 0.5 mL soil homogenate solution, and an equal volume of substrate. Control wells were employed as described above with the exception that soil homogenate was utilized in place of tyrosinase buffer solution. The pH conditions were manipulated as described above. Upon completion of the reaction, the plates were centrifuged for 5 minutes at 4000 rpm to remove particles and quench the reaction. A 235 uL aliquot was taken from the top of each well and absorbance was measured as in the tyrosinase pH range experiment.

Phenol oxidase assay protocol, field site comparisons:

Phenol oxidase assays were carried out on replicate core samples from each of the Minnesota field sites across season and depth under in situ pH. One gram of peat was homogenized in 50 mL of 50 mM acetate buffer at environmental pH. Ratios and controls were consistent with the soil enzyme pH range experiment.

Extinction coefficients for substrates were determined empirically through the use of a regression of fully oxidized substrate concentration and their absorbances. Several concentrations of ABTS and L-DOPA were brought to full oxidation with use of purified PO enzyme and a regression was created to determine the values for each substrate's extinction coefficient (11667 and 7415 for ABTS and L-DOPA, respectively).

To ensure an accurate measurement of enzyme activity a time series must be conducted for enzyme assays in environmental samples and for standard enzyme samples. Measurements taken at the nonlinear time points will lead to underestimation of enzyme activity. It is recommended to have enough time points to find the duration of time at which the reaction is no longer linear. Once kinetics of the enzymes present has been assessed, all samples must be run within linear rates of oxidation to calculate enzyme activity. If additional time points are utilized they will present a clearer picture of the rates of activity of bulk soil enzymes. The duration of the assay depends on kinetics assessed in the initial steps of the experiment and varies on a site-by-site basis. Net absorbance between two time points within linear ranges were taken in order to obtain rates in all samples and experimental conditions. Utilizing two time points in this manner reduces the amount of within sample variation. All samples were assayed in 4 - 7 pH conditions and with basic differences such as homogenization method, buffer, substrate concentration and other parameters until optimal conditions were established. All assays were run for 1 hour and the difference in activity was assessed from 15 to 60 minutes to reduce affects of lag and variation within core measurements. Wet to dry weight ratios were determined with 1 gram of peat in triplicate by heating to a stable mass at 60 ^{o}C. Statistical comparisons were conducted using a two-tailed T test.

RESULTS:

A pronounced pH effect was observed in all experiments with standard enzyme as well as with wetland soils. Both the rate and extent of substrate oxidation were impacted by pH. The substrates tested, L-DOPA and ABTS, showed a different and generally converse response to pH.

Calibration of phenol oxidase assay with the standard enzyme, tyrosinase:

Tyrosinase oxidized L-DOPA rapidly, with activity becoming nonlinear within minutes of the initiation of the reaction (Figure 1.1). In contrast, when ABTS was used as the substrate, the reaction was slower, with nonlinear rates of oxidation achieved after a few hours (Figure 1.2, Table 2). Assay substrates also showed very different pH thresholds of oxidation with little to no oxidation of L-DOPA and ABTS by tyrosinase observed at pH 4 and pH 7, respectively. With L-DOPA, activity decreased by up to two orders of magnitude at pH 4 relative to that observed at a higher pH of 5 to 6. With ABTS, activity at pH 4 was six and ten times higher in comparison to that observed at pH 5 and 6, respectively.

	Site ID	pH 4	pH 5	pH 6	pH 7
ABTS	319 Swamp	2.1±0.18	1.11±0.33	0.98±0.52	1.87±0.24
	Center Swamp	4.41±1.24	3.05±0.65	2.66±0.21	0.32±1.2
	Panacea Marsh	0.42±0.23	0.96±0.26	0.92±0.12	0.57±0.32
	S1 bog	1.25±0.11	0.95±0.17	0.70±0.21	0.62±0.11
	Bog Lake Fen	0.55±0.15	0.29±0.177	0.21±0.20	0.054±0.19
	Tyrosinase	76.77±4.88	35.25±2.65	12.01±8.11	0.0±0.0
L-DOPA	319 Swamp	0.08±0.24	1.0±0.22	2.54±1.03	5.80±2.67
	Center Swamp	0.51±0.65	0.76±0.67	3.58±0.64	3.08±.068
	Panacea Marsh	0.47±0.22	0.62±0.29	1.47±0.67	2.35±0.62
	S1 bog	0.11±0.10	0.17±0.21	0.57±0.35	1.19±0.34
	Bog Lake Fen	0.14±0.16	0.058±0.17	0.48±0.40	0.20±0.26
	Tyrosinase	0.34±0.11 *10^6	11.59±0.45 *10^6	26.46±1.66 *10^6	27.80±1.13 *10^6

Table 2: Phenol oxidase activity as a function of pH and assay substrate in reactions with standard enzyme and in wetland soils. Activity is represented as μmol per g dry peat or mg tyrosinase per minute.dry

Calibration of phenol oxidase assay in wetland soils across a range in soil pH:

In general, similar trends were observed in wetland soils (Figures 2.1 to 2.5, Table 2) in comparison to experiments conducted with tyrosinase, with one exception: in soils of Bog Lake fen, a maximum in PO activity was observed with L-DOPA at neutral pH. Conversely, with the exception of soils from the pH 7 site at Panacea Marsh, maxima in PO activity were observed with ABTS at pH 4. A slightly different pH threshold was observed for L-DOPA oxidation in wetland soils in comparison to the tyrosinase experiments. From pH 6 to 5, activity measured with L-DOPA largely decreased ($p < 0.0001$) with tyrosinase (*Figure* 1.1). At Center Swamp activities with L-DOPA increase at pH 6 compared to pH 5 ($p < 0.006$). In contrast, a linear increase in activity was observed with pH increase from in situ conditions at S1 bog (*Figure* 2.4) with activity

increasing at pH 5 with respect to pH 4 ($p < 1E-5$). The pH response of ABTS oxidation in wetland soils showed more variation. At Panacea Marsh and 319 Swamp, ABTS oxidation did not change substantially as a function of pH. Center swamp and S1 bog however showed an overall decrease in activity with pH with ABTS as a substrate. The activity exhibited a marked decrease at pH 6 from pH 5 in Center swamp (*Figure* 2.1; $p < 0.006$), where S1 showed even more sensitivity with a significant decrease at pH 5 from pH 4 (*Figure* 2.5; $p < 0.02$). Bog Lake fen displayed significant differences in activity at all pH increments with ABTS as a substrate ($p \leq 0.004$ at all increments).

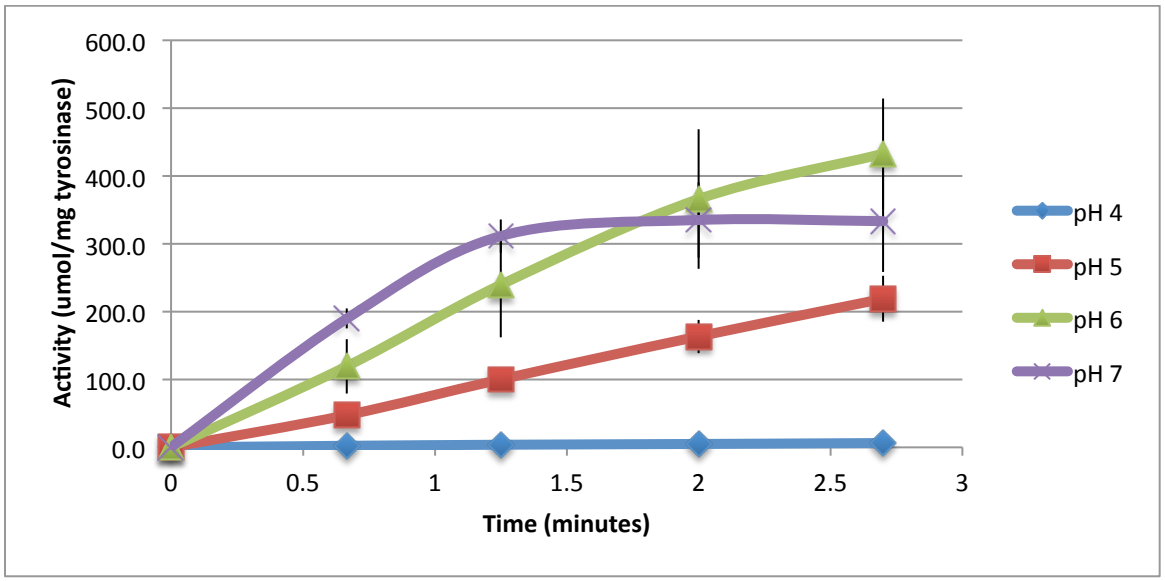

Figure 1.1: Time course of tyrosinase activity as a function of pH. Activities were determined using L-DOPA as the assay substrate. Values represent the average extent of oxidation at each time point +/- one standard deviation.

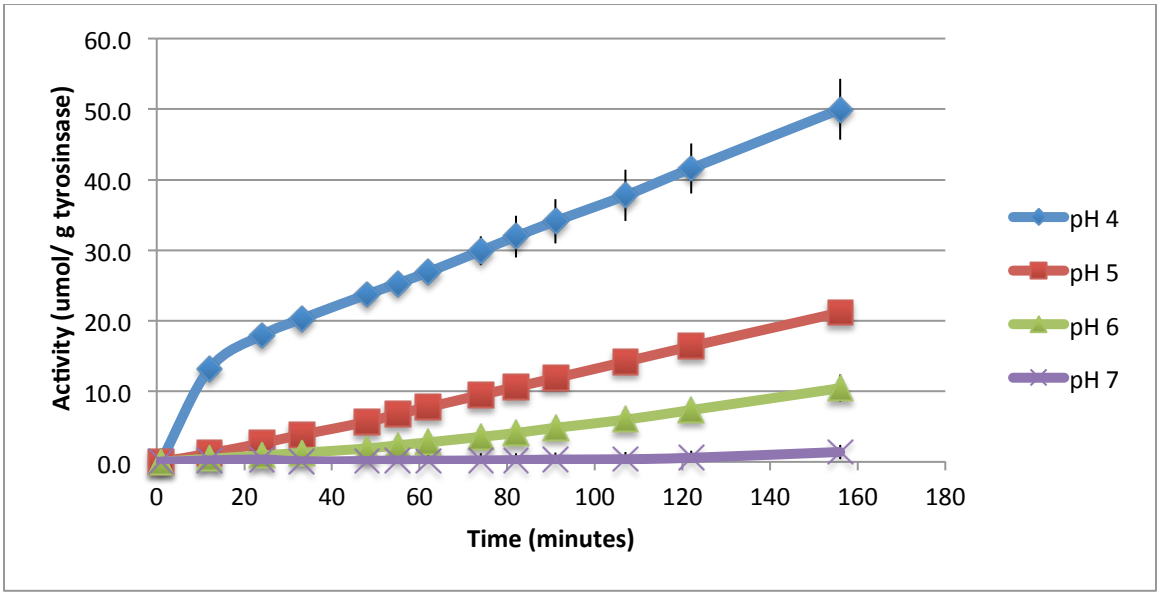

Figure 1.2: Time course of tyrosinase activity as a function of pH. Activities were determined using ABTS as the assay substrate. Values represent the average extent of oxidation at each time point +/- one standard deviation.

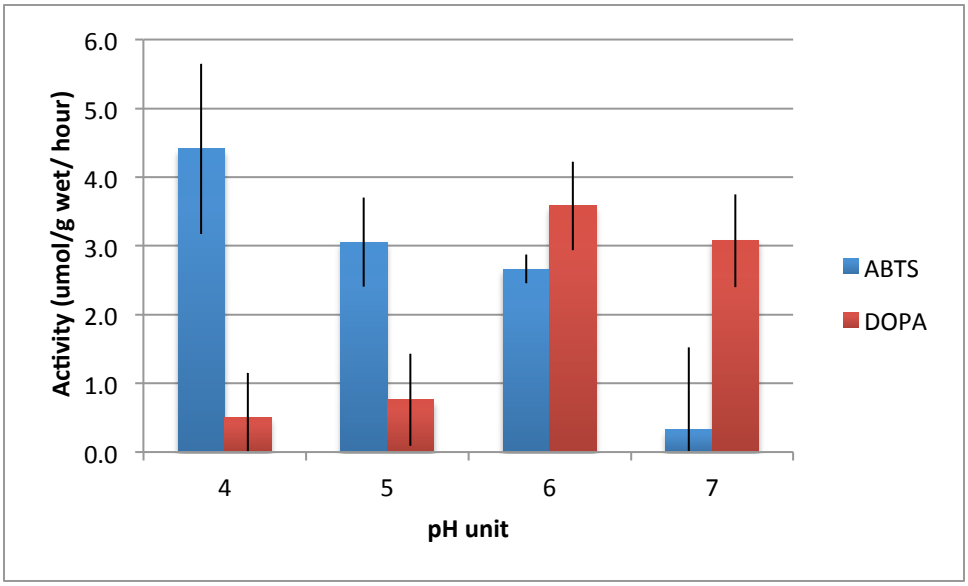

Figure 2.1: Phenol oxidase activity as a function of pH in soil samples collected from the Center Swamp. Activities were determined using ABTS (blue) or L-DOPA (red) as the assay substrate. Values represent the average extent of oxidation +/- one standard deviation. Averages were obtained in triplicate cores.

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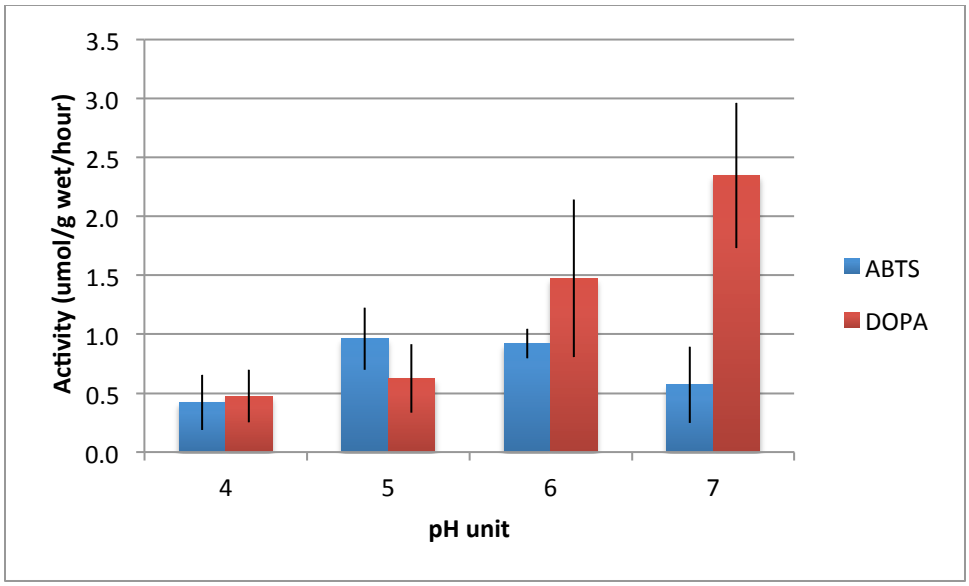

Figure 2.2: Phenol oxidase activity as a function of pH in soil samples collected from the Panacea Marsh. Activities were determined using ABTS (blue) or L-DOPA (red) as the assay substrate. Values represent the average extent of oxidation +/- one standard deviation. Averages were obtained in triplicate cores.

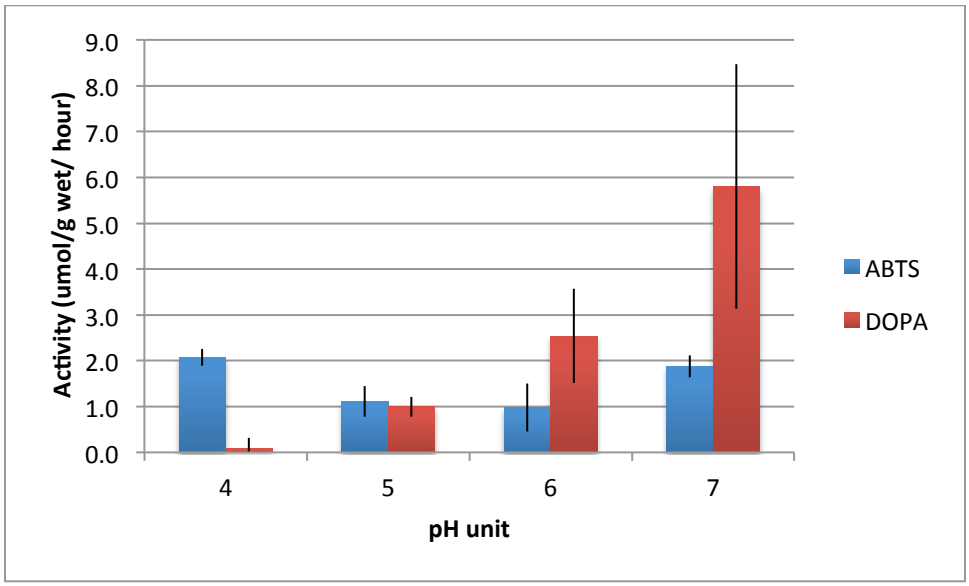

Figure 2.3: Phenol oxidase activity as a function of pH in soil samples collected from the 319 Swamp. Activities were determined using ABTS (blue) or L-DOPA (red) as the assay substrate. Values represent the average extent of oxidation +/- one standard deviation. Averages were obtained in triplicate cores.

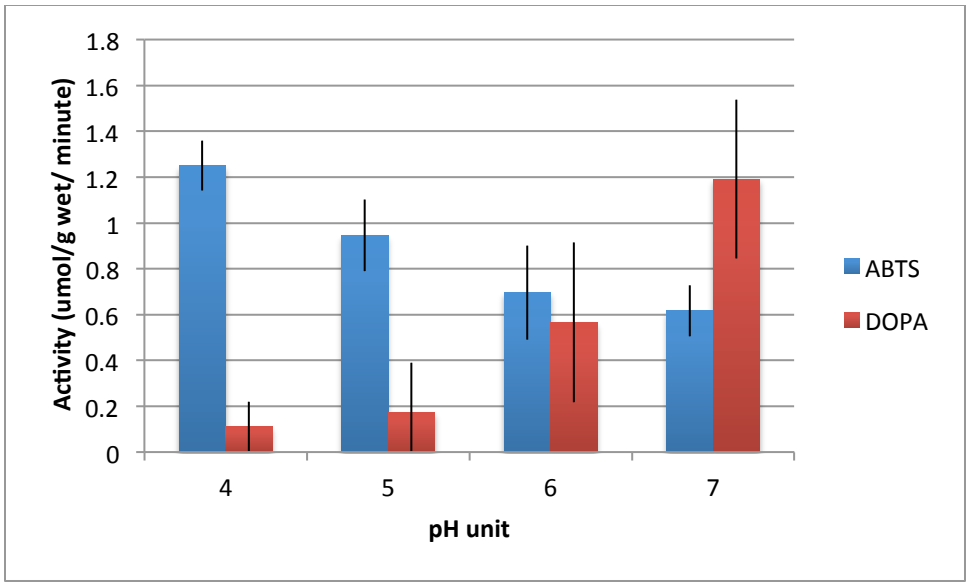

Figure 2.4: Phenol oxidase activity as a function of pH in soil samples collected from the S1 bog. Activities were determined using ABTS (blue) or L-DOPA (red) as the assay substrate. Values represent the average extent of oxidation +/- one standard deviation. Averages were obtained in triplicate cores.

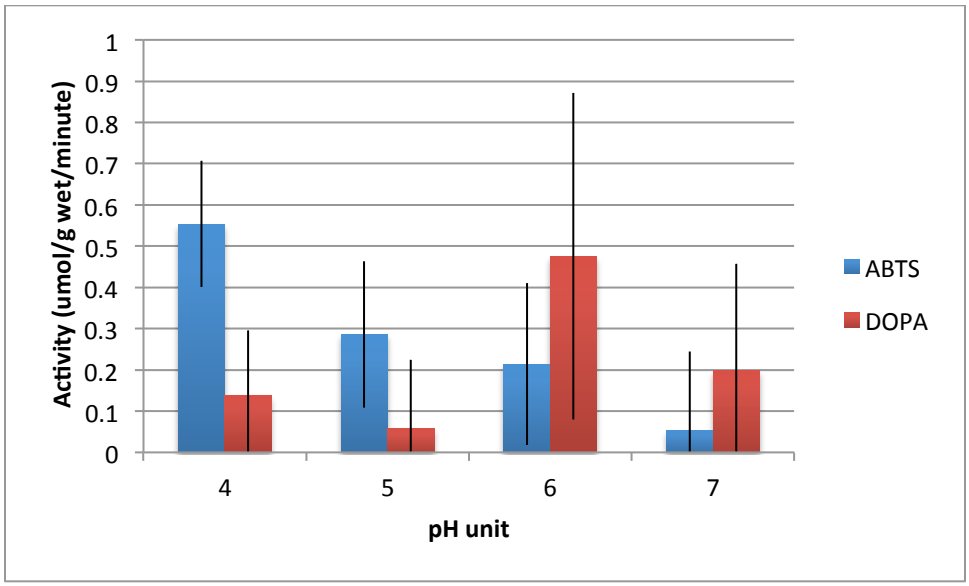

Figure 2.5: Phenol oxidase activity as a function of pH in soil samples collected from the Bog Lake fen. Activities were determined using ABTS (blue) or L-DOPA (red) as the assay substrate. Values represent the average extent of oxidation +/- one standard deviation. Averages were obtained in triplicate cores.

Field data:

Calibration results indicated that acidic wetland soils with a pH of less than 5 oxidize ABTS preferentially to L-DOPA. Therefore, ABTS was employed to quantify PO potential activity in Minnesota peatlands with an in situ pH of ~4 (Figures 3.1 to 3.3). A similar range in PO activity was observed at all sites sampled in peatlands. In S1 bog, Bog Lake fen and Zim bog activity attenuated with depth. Activities measured deep in the peat column (50-100 cm depth) were on average 10 to 50 times lower than those measured at the surface (0-20 cm depth). In the mid-depth interval (30-50 cm depth), elevated activity was observed at the fen site in comparison to the bogs.

Porewaters were sampled over the same depth range at Bog Lake fen, S1 and Zim bog. In general, one to two orders of magnitude lower rates of PO activity were measured in the porewater and no trends were observed between replicate sites and depths (Table 3). In addition, it should be noted that little to no PO activity could be detected in porewater using a similar incubation time to that used for solid peat. Thus, incubations to determine PO activity in porewater were conducted over a 24 to 48 hour period.

Lastly, the impact of soil sampling method on PO activity was determined at the S1 bog site during the summer field sampling. No significant difference was observed in PO activity determined using the relatively non-destructive S1 summer claw (*Figure* 3.7) in comparison to the destructive peat borer summer samples (*Figure* 3.1) ($p > 0.058$).

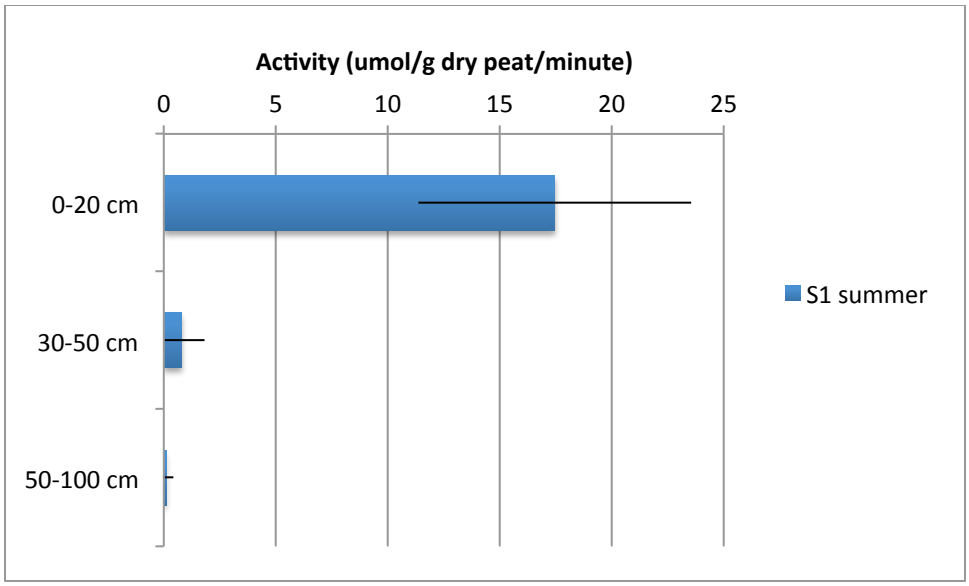

Figure 3.1: Phenol oxidase activity as a function of depth in soil samples collected from the S1 bog in July 2013. Activities were determined using ABTS as the assay substrate at in situ pH. Values represent the average extent of oxidation per minute +/- one standard deviation. Averages were obtained quintuplicate cores.

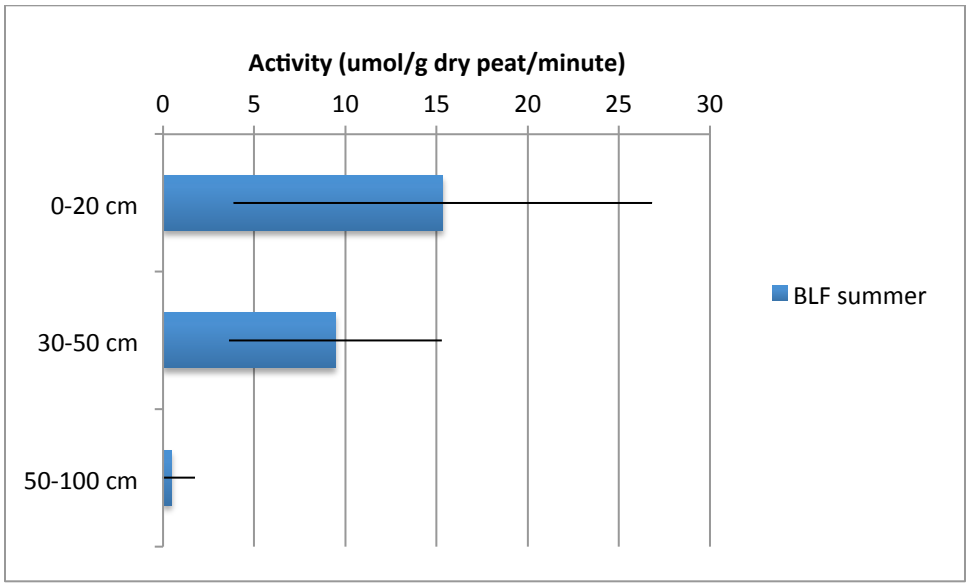

Figure 3.2: Phenol oxidase activity as a function of depth in soil samples collected from Bog Lake fen in July 2013. Activities were determined using ABTS as the assay substrate. Values represent the average extent of oxidation per minute +/- one standard deviation. Averages were obtained quintuplicate cores.

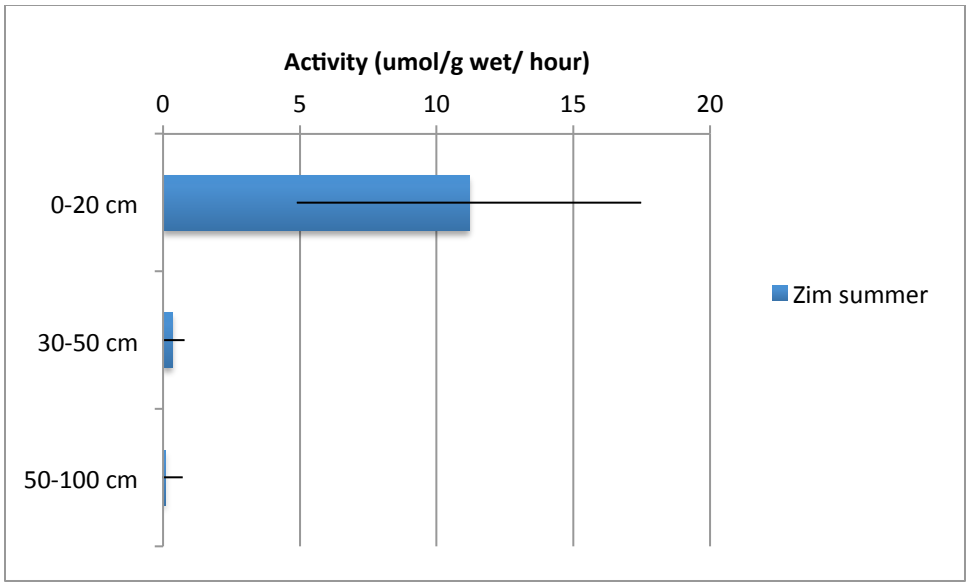

Figure 3.3: Phenol oxidase activity as a function of depth in soil samples collected from Zim Bog in July 2013. Activities were determined using ABTS as the assay substrate at in situ pH. Values represent the average extent of oxidation per minute +/- one standard deviation. Averages were obtained quintuplicate cores.

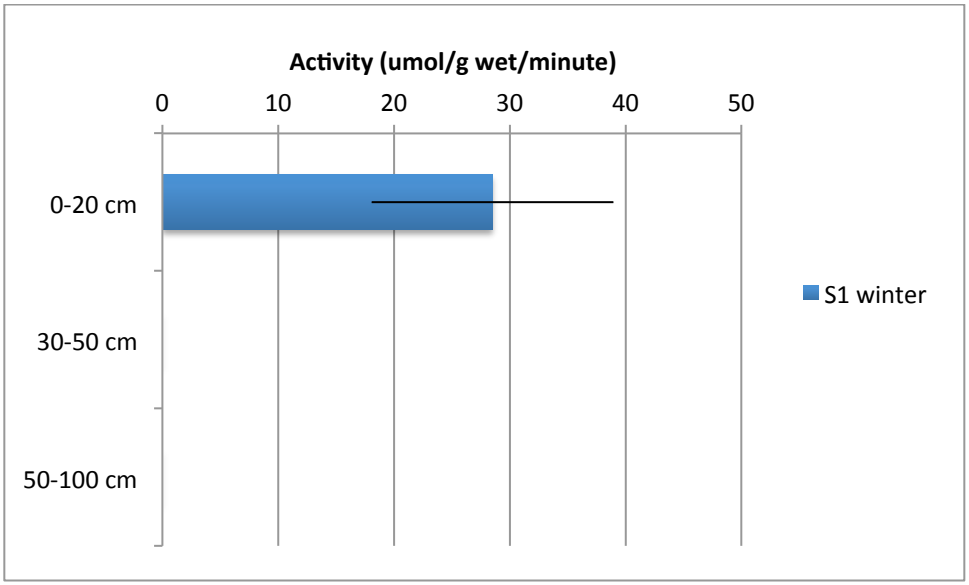

Figure 3.4: Phenol oxidase activity as a function of depth in soil samples collected from the S1 bog in April, 2014. Activities were determined using ABTS as the assay substrate at in situ pH. Values represent the average extent of oxidation per minute +/- one standard deviation. Averages were obtained quintuplicate cores.

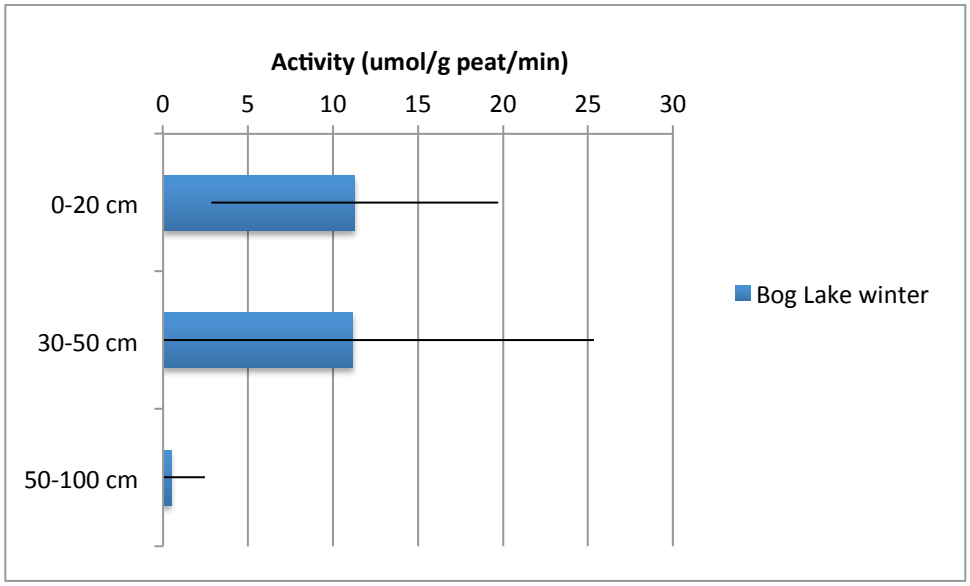

Figure 3.5: Bog Lake fen April 2014 phenol oxidase activity as a function of depth in soil samples. Activities were determined using ABTS as the assay substrate at in situ pH. Values represent the average extent of oxidation per minute +/- one standard deviation. Averages were obtained quintuplicate cores.

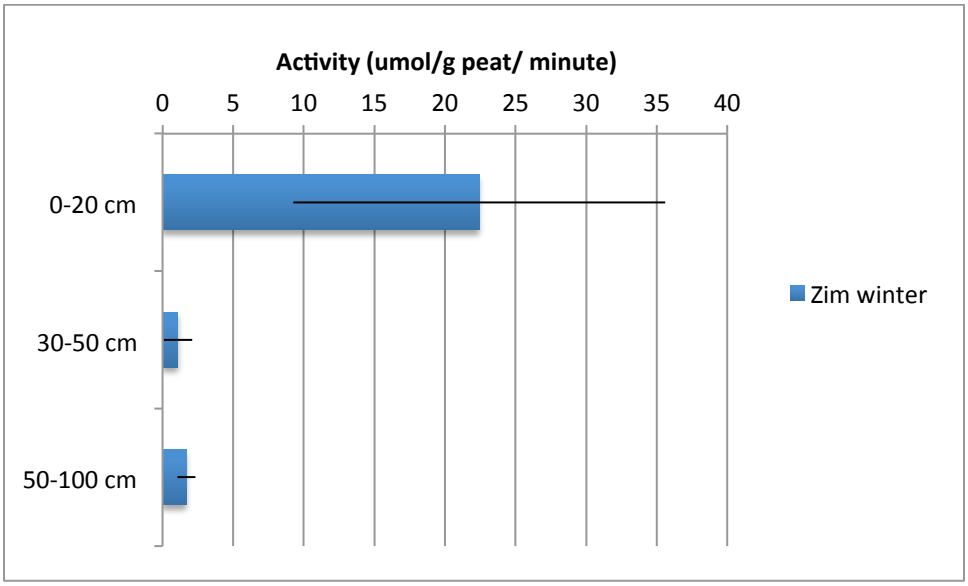

Figure 3.6: Phenol oxidase activity as a function of depth in soil collected from the Zim bog in April 2014. Activities were determined using ABTS as the assay substrate at in situ pH. Values represent the average extent of oxidation per minute +/- one standarddeviation. Averages were obtained quintuplicate cores.

Depth	S1 Bog	Bog Lake fen	Zim bog
25 cm	0.041±0.001	0.12±0.01	0±0
50 cm	0±0	0.16±0.01	0.052±0.0
75 cm	0±0	0.07±0.00	-
100 cm	0.022±0.001	0.015±0.00	0.057±0.0
150 cm	0±0	0.245±0.05	0.049±0.0
200 cm	0.088±0.002	0.0±0.0	-

Table 3: Pore water phenol oxidase activity (µmol/ mL/ day) as a function of depth in samples collected during July of 2013. Activities were determined using ABTS as a substrate at in situ pH. Averages were obtained in triplicate.

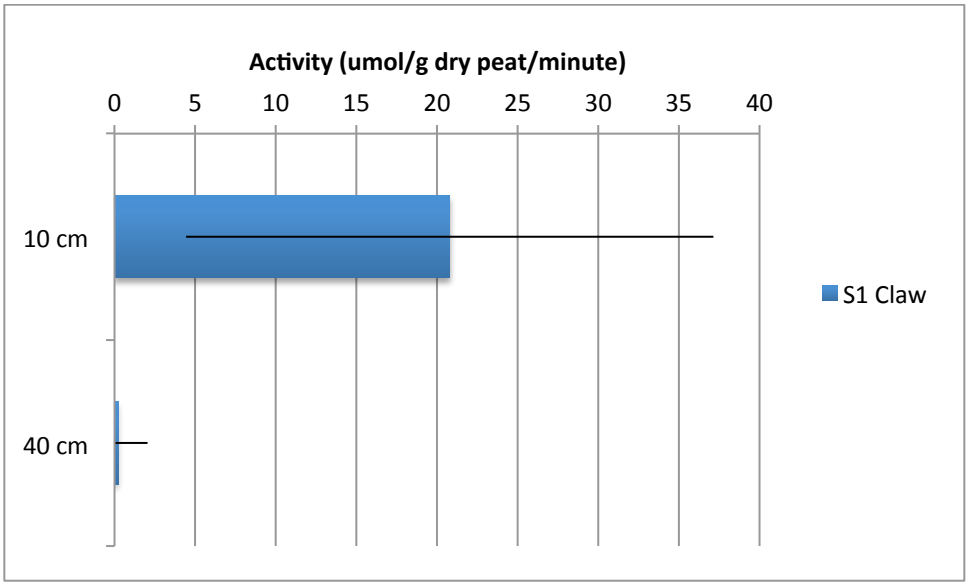

Figure 3.7: phenol oxidase activity in the S1 bog as a function of depth using the claw sampling method and ABTS as a substrate. Values represent the average extent of oxidation per minute +/- one standard deviation. Averages were obtained in triplicate cores.

	Activity (umol/dry peat g/ minute)		
summer activity	0-20 cm	30-50 cm	50-100 cm
S1 bog	17.46±6.09	0.79±1.03	0.12±0.31
Bog Lake Fen	15.35±11.48	9.46±5.83	0.48±1.27
Zim bog	11.19±6.30	0.34±0.45	0.08±0.638
S1 Claw	20.79±16.34	0.23±1.82	
	Activity (umol/ dry g/ minute)		
winter activity	0-20 cm	30-50 cm	50-100 cm
S1 bog	28.51±10.44	0.0±0.0	0.0±0.0
Bog Lake Fen	11.25±8.44	11.14±14.32	0.48±0.95
Zim bog	22.43±13.18	1.10±1.00	1.68±0.64

Table 4: Phenol oxidase activity quantified as a function of depth and season in soils of northern Minnesota peatlands.

Zim bog (Figure 3.3, Figure 3.6) showed similar trends with depth to that of S1, but potential PO activity was significantly lower compared to S1 at 0-20 cm in winter months ($p < 0.0003$) and in summer months ($p < 0.0002$) with no difference between activities at other depths. Bog Lake fen was the only site to show elevated activity at depths below surface. At both Zim and S1 bog sites, significantly higher activity was observed during the winter ($p < 0.00001$) in comparison to the summer ($p < 0.03$) in more shallow depths, while there was no detectable change below the surface. No significant difference in potential PO activity was observed across seasons at Bog Lake fen (0-20 cm $p < 0.9$; 30-50 cm $p < 0.08$; 50-100 cm no trend; Table 4).

Discussion:

Impact of pH on the phenol oxidase enzyme assay:

Extensive studies of PO activity were conducted using the L-DOPA method in peatlands over the past two decades (Pind et al., 1994; Williams et al, 2000; Freeman et al., 2001; 2004; Fenner and Freeman, 2011). Indeed, Freeman et al. (2001) used the L-DOPA method to put forth the "enzyme latch" hypothesis, which serves as a major paradigm in the microbiology and biogeochemistry of peatlands. The "enzyme latch" hypothesis states that organic matter decomposition in peats is controlled by plant-derived polyphenolic compounds which act to inhibit overall microbial metabolism. Thus, this single class of enzymes, phenol oxidases, plays a central role in the release of greenhouse gases in peatlands (Limpens et al., 2008), which are believed to store nearly one-third of all soil carbon on a global scale (Gorham et al., 2012). However, the results of previous work may in some cases be called into question due to the adverse effects of pH on the PO assay. Although pH has long been implicated as a control of oxidative enzyme activity (German et al., 2011), previous studies in peatlands were often not conducted at in situ pH and did not validate or calibrate the PO assay to account for pH effects.

L-DOPA is the most commonly utilized substrate for measurement of oxidative enzyme activity in soils and sediments (Sinsabaugh, 2010). The majority of measurements of PO activity have been conducted in upland soils which are not saturated with water and at a pH of ~5 which is close to environmental pH in these soils (Bach et al., 2013; Eichlerova et al., 2013). In many soil and leaf litter systems, L-DOPA is an adequate assay substrate. However, recent work showed that the L-DOPA method may

result in unrealistic PO activities when assay pH deviates from the in situ pH of the soil, especially in wetlands such as peatlands that have an in situ pH of < 5 (Tahvanainen and Haraguchi, 2013). Results from this study corroborate those of previous work insofar that pH is demonstrated to have dramatic effect on the activity of PO enzymes.

In the field, this study focused on soils collected from northern Minnesota peatlands. Preliminary measurements using L-DOPA as an assay substrate in peatland soils showed large variations in PO activity which did not reveal any meaningful ecological trends similar to past work conducted with L-DOPA in acidic peatland soils (Reiche et al., 2009). Since the introduction of the ABTS method by Floch et al. (2007), ABTS oxidation has been used as an assay for PO activity, especially in acidic soils (Eichlerova et al., 2013). However, this method has not been applied extensively or verified for use in wetland soils (Jassey et al., 2012). Thus, this study set out to verify and calibrate a method for use in wetlands, focusing on peatland systems which generally contain an in situ pH of < 5 (Tahvanainen and Haraguchi, 2013).

The results of this study indicate that the oxidation of substrates commonly used to quantify phenol oxidase activity, L-DOPA and ABTS, are strongly pH dependent in assays with the standard enzyme tyrosinase and in wetland soils. A threshold in substrate oxidation was observed at pH 5. The PO assay is sensitive and activity could be detected with either substrate across a pH range of 4 to 7. However, with the standard enzyme tyrosinase, it was shown that a large change in oxidation rates occurred at pH 5. At pH < 5, L-DOPA oxidation rates were greatly diminished and ABTS oxidation was at a maximum. Above pH 5, ABTS oxidation occurred at much slower rates and L-DOPA oxidation was at a maximum. Experiments in wetland soils corroborated observations

made with tyrosinase. Thus, ABTS is recommended to be an effective substrate for the quantification of PO activity at an in situ pH of < 5, while L-DOPA is recommended at an in situ pH of > 5.

Application of modified assay to peatlands of northern Minnesota:

The modified ABTS method was applied extensively to two bog sites (S1, Zim) and one fen site (Bog Lake fen) in northern Minnesota. The S1 bog site is located in the Marcell Experimental Forest (MEF) in northern Minnesota where the US Department of Energy's Oak Ridge National Laboratory and the USDA Forest Service are conducting a large scale field climate manipulation known as "Spruce and Peatland Response Under Climatic and Environmental Change" (SPRUCE). The overall goal of this research is to understand carbon turnover and greenhouse gas release at the SPRUCE site and therefore PO activity was tested more extensively in S1 bog. For example, PO activity was investigated in peat porewater in order to develop a method of monitoring activity repeatedly in a nondestructive manner. Activities observed in peat porewaters collected from surface depths at S1 bog ranged from 0.025 to 2.56 μmol mL^{-1} day^{-1} and were one to two orders of magnitude lower than activities observed at the same depth in solid peat. Further, PO activity in porewater showed large variations, even when collected at the same site, and no ecologically meaningful trends could be observed (Table 3). The results indicate that the majority of PO activity is associated with the solid peat and not in the porewater. Activities measured in the porewater were close to the detection limit, which may explain why meaningful trends were not observed and why porewater may not provide a reliable determination of PO activity.

Potential rates of PO activity in solid peat were compared at two ombrotrophic bogs,

which receive the majority of nutrient input from precipitation, and at a minerotrophic fen, which receives groundwater nutrient input. At all sites, PO activity was highest at the surface (0-20 cm depth) and decreased rapidly with depth to 100 cm. At the bog sites, rates attenuated more rapidly with peat depth, resulting in an order of magnitude lower rates at 30-40 cm depth. In contrast, PO activities measured at 30-40 cm depth in the fen were nearly as high as those measured at the surface. The fen also differs from the bogs in that vascular plants such as sedges (Carex) were much more abundant. Since PO activity is dependent on the supply of molecular oxygen, elevated activities at depth in the fen could be explained by the input of oxygen from vascular plant roots.

Multiple lines of evidence support these trends of PO activity with depth at the S1 bog and Bog Lake fen sites of MEF. Strong vertical stratification has been observed in biogeochemical and microbiological parameters that points to peat decomposition (Tfaily et al., 2014; Lin et al., 2014a&b). For example, trends in PO activity were corroborated by potential rates of organic matter decomposition measured as CO_2 production in microcosms at the same depths and sites in the S1 bog (Tfaily et al., 2014). Potential rates of PO activity were 10 to 50 times higher at the surface in comparison to the deep peat, similar to changes in decomposition rates. Activities of seven enzymes that mediate the mineralization of carbon, nitrogen, and phosphorus were all quantified in a recent study of the S1 bog (Lin et al., 2014). Similar to the potential PO activities measured here, activities of the other enzymes decreased with depth; many showed a decrease of approximately one order of magnitude from the surface to the deep peat. Thus, PO is localized to the surface and activity parallels that of other extracellular enzymes.

Surprisingly, the highest potential PO activity was observed during the winter at both

S1 and Zim bogs, which is contrary to the hypothesis that enzyme activity would be reduced in winter months. During the colder sampling period in April, the peatland was partially frozen and many areas had recently thawed. Thus, elevated PO activities may have been the result of a thawing affect that caused more of the enzymes to be released. The seasonal changes in PO activity that we observed were small, and other extracellular enzymes did not show a strong seasonal effect at the same Minnesota peatland sites (Lin et al., 2014). Further studies are needed to elucidate the biogeochemical controls of oxidative enzyme activity in peatlands.

Conclusions:

In this study, ABTS and L-DOPA oxidation were shown to be strongly pH dependent in experiments with wetland soils and with the standard enzyme tyrosinase. A threshold was revealed at pH 5 below which L-DOPA oxidation is largely diminished. Conversely, ABTS oxidation of tyrosinase and PO in wetland soils showed maximum activity at pH 4. Thus, ABTS is suggested as an effective substrate for the quantification of PO activity in wetlands, such as boreal peatlands which often contain an in situ pH of less than 5. At an in situ pH above 5, L-DOPA is likely to be a more effective substrate for the quantification of PO activity. However, activity with these substrates is site-specific and extensive calibration is recommended for each new site. In the field, the majority of PO activity in peatland soils was shown to be associated with solid phase peat and not in the porewater. Rates of phenol oxidase activity at near in situ pH were shown

to covary with the activity of other enzymes and with peat decomposition performed in previous studies of the field sites (Tfaily et al 2014, Lin et al 2014a and b).

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